“Intracellular Calcium Responses of Endothelial Cells Exposed to Pulsatile and Oscillatory Fluid Mechanical Shear Stresses”

Undergraduate Honors Thesis

Presented in partial fulfillment for the Bachelor of Science with honors research distinction at

The Ohio State University

By

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Graduating May 7, 2017

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Abstract

Calcium (Ca^{2+}) is a ubiquitous 2nd messenger that dictates many cellular pathways including muscle contraction, cell proliferation, and apoptosis. Disregulation of Ca^{2+} homeostasis is believed to contribute to vascular endothelial cell (EC) dysfunction, which transitions the endothelium to a pro-inflammatory mode, initiating atherosclerosis. Since ECs are continually exposed to fluid mechanical shear stress from blood flow, the effect that shear stress has on the intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) is of major interest. Increases in fluid shear stress are known to increase cytosolic Ca^{2+}. In ECs, the endoplasmic reticulum (ER), the mitochondria, and the extracellular media are the three main sources of Ca^{2+} attributed to this response. Their relative contribution to the $[Ca^{2+}]_i$ response under steady laminar shear stress was investigated previously in our laboratory, but this flow profile is not experienced in human arteries. This study aims to expand upon our earlier published work by investigating the shear-induced $[Ca^{2+}]_i$ response of cultured ECs to differing physiological flows, specifically pulsatile (10±5 dyn/cm^2) and oscillatory (0.1±5 dyn/cm^2) laminar flows. To monitor the Ca^{2+} response to flow, ECs were incubated with the Ca^{2+}-sensitive probe Fluo-4, and then sheared in the presence of either pulsatile or oscillatory flow. Based on normalized fluorescence responses corresponding to $[Ca^{2+}]_i$ responses, oscillatory flow was found to exhibit lower peak magnitudes, higher % of oscillating cells, and no synchronization at shear onset compared to pulsatile flow. This study quantitatively characterized the $[Ca^{2+}]_i$ responses under both flow profiles and determined significant differences between them. Further experimentation researching the $[Ca^{2+}]_i$ mechanisms responsible for the contrasting $[Ca^{2+}]_i$ responses is still needed. A greater understanding of these phenomena could lead to better drug development to prevent and/or treat endothelial dysfunction, and ultimately delay or reverse cardiovascular disease.

Acknowledgements

I would like to acknowledge the invaluable guidance, oversight, and encouragement provided by my research advisor, Dr. B. Rita Alevriadou, PhD, Associate Professor, Biomedical Engineering and Internal Medicine (Cardiovascular Medicine); the training, experimental processes, and prior work of Dr. Christopher G. Scheitlin, PhD, a former student in our lab; the assistance with data analysis and figure generation by Mr. Akshar Patel, the current MS student in our lab; Dr. Jaspreet Sachdeva, the current Postdoctoral researcher in our lab and other past lab members whose work laid the foundation for my own. I would like to acknowledge my family and friends who have supported me throughout my research endeavors. I would finally like to acknowledge the funding by NIH, AHA, and OSU (the latter in the form of the Undergraduate Education Summer Research Fellowship, Research Scholars Award, National Buckeye Scholarship, Trustees Scholarship, Wening Scholarship, STEP Scholarship, and Undergraduate Honors Thesis Scholarship), and Drs. Alevriadou and Gooch for serving as members of my thesis defense committee.

CURRICULUM VITAE

EDUCATION:

The Ohio State University – Columbus, OH
Major • Biomedical Engineering GPA • 3.80/4.00 Expected Graduation • May 2017

RESEARCH EXPERIENCE:

Cardiovascular Bioengineering/VMOS Lab **The Ohio State University** **2015-Present**

- Pursuing an independent research thesis with Honors Research Distinction in Dr. B. Rita Alevriadou's Vascular Mechanotransduction & Oxidative Stress (VMOS) Laboratory sponsored by a scholarship from the Ohio State College of Engineering
- Investigating intracellular calcium dynamics in cultured vascular endothelial cells exposed to fluid mechanical shear stress (specifically, the calcium exchange between the endoplasmic reticulum and the mitochondria)
- Exposing endothelial cells to different shear stress profiles and chemical agonists to better understand the molecular mechanisms that lead to onset of cardiovascular disease/atherosclerosis
- Skills: Aseptic technique, cell culture, fluorescence microscopy, video intensity analysis (ImageJ, CellProfiler, MATLAB), syringe pump calibration, shear stress experimentation, and calcium homeostasis mathematical modeling

Nanotechnology Project **The Ohio State University** **Spring 2014**

- Designed a lab-on-a-chip through SolidWorks that analyzes fluorescein levels in tears to diagnose dry eye syndrome
- Coordinated team meetings, organized project notebook, wrote lab reports, practiced good lab procedure
- Accomplished goal of creating a functional and accurate microfluidics chip verified through calibration and testing

PRESENTATIONS AND CONFERENCES:

- BMES Annual Meeting (10/8/16 Minneapolis, MN)
  - Presented an undergraduate research poster titled "Ca^{2+} Response in Endothelial Cells Exposed to Different Flows" by Alexander D. Cetnar, Akshar Patel, Christopher G. Scheitlin, and B. Rita Alevriadou
- OSU Fall Undergraduate Research Forum (9/15/16 Columbus, OH)
  - Presented an undergraduate research poster titled "Intracellular Calcium Signaling in Endothelial Cells Exposed to Different Flows" by Alexander D. Cetnar, Christopher G. Scheitlin, and B. Rita Alevriadou
- BMES Annual Meeting (10/9/15 Tampa, FL)
  - Presented 20-minute mentorship best practices speech to colleagues of BMES student chapters across the US
- BMES Midwest Regional Meeting (11/6/15 Akron, OH)
  - Attended conference with lab members and BMES officers to network with other faculty and professionals

COURSEWORK AND SKILLS:

Relevant Coursework: Tissue Engineering, Biomaterials, Nanotechnology Engineering, Senior Capstone Design, Independent Thesis Research, Quantitative Physiology, Measurements & Instrumentation, and Numerical Simulations

Computer Skills: MATLAB, SolidWorks, COMSOL, ImageJ, Cell Profiler, ReplicatorG, ChemDraw, WordPress, Minitab, MacOS

SCHOLARSHIPS AND AWARDS:

- Undergraduate Education Summer Research Fellowship
- Dean's List for all semesters (AU13-SP16)
- Research Scholars Awardee
- National Buckeye Scholar
- Trustees Scholar
- Wening Scholar
- STEP Scholar

Alpha Eta Mu Beta **The Ohio State University** **2015-Present**

- Social Chair: coordinating social events and outings for current members and heading future member recruitment
- Recognized member of this BME Honorary Society for my academic status (GPA in the top 1/5 of my major class)
- Promoting professional development, unity of the BME student body, and a closer student-faculty relationship

LEADERSHIP:

Ohio State Biomedical Engineering Society **The Ohio State University** **2013-Present**

As President of a 115-member student chapter and leader of a 9 officer executive board (2015-2016):

- Awarded:
  1) Chapter Outstanding Mentoring Program Award - National BMES
  2) Outstanding Chapter Industry Program Award - National BMES
  3) Outstanding Partnership with Industry Award – The Ohio State University College of Engineering
- Coordinated blood drives partnered with American Red Cross to yield over 150 units of blood
- Led meetings, programs, and conferences to promote professional development, career opportunities, and service
- Networked with BME faculty, companies, and organizations as well as students in and outside the major

As Interim Treasurer and Webmaster (2014-2015):

- Managed a budget of ~$10,000 involving audit, fundraising, and sponsorship oversight
- Developed and maintained the organization website via WordPress hosting and headed event digital advertisement

As Freshman Mentorship Coordinator (2013-2014):

- Planned events (social, professional, and volunteer) for BMES members, specifically targeting freshmen BME class

Resident Advisor **The Ohio State University** **2014-Present**

- Promoting academic excellence, addressing student needs, and encouraging involvement for over 250 residents to support the mission of Residence Life at The Ohio State University and Columbus as a Columbus Ambassador
- Organizing student-focused programs, managing floor budgets ($200-500), and fostering community among residents
- Cooperating with co-workers and supervisors in team-oriented environments to meet programming deadlines

EXPERIENCE:

BME International Summer Design Intern **The Ohio State University** **Summer 2016**

- Optimized final models of incomplete, medically-related Senior Capstone Projects alongside a 10-member team
- Modified a hemi side walker to lessen user strain and discomfort while improving stability with students from Nanjing University (China) by machining, 3D-printing, and building components during a summer design experience

Digital Advertiser Intern **Columbus, OH** **Summer 2015**

- Interned with Print Syndicate LLC as a digital advertiser and marketer to advance sales and merchandise exposure
- Promoted products via social media by creating, tracking, and evaluating ads while managing sizable spreadsheets
- Expanded the number of followers and influencers, optimized profits and data reporting, and enhanced Excel skills

SERVICE:

Cru at Ohio State - RealLife **The Ohio State University** **2015-Present**

- Bible Study Leader: leading a weekly Bible study in my residence hall to promote and grow in Christian fellowship
- Worship Band Drummer: performing and rehearsing weekly as the worship band drummer for audiences of 200-400

Global Together Foundation **Columbus, OH** **2016-Present**

- Mentoring and empowering others through a "pay-it-forward" mentality to establish a local and global community
- Collaborating with a local non-profit, 1DivineLine2Health, to transport medical crates to Syrian refugees in need

Engineering Without Borders **The Ohio State University** **2013-2014**

- Performed local service projects that are engineering-related to benefit the community with the OSU local chapter
- Planned activities to introduce middle and high school students to engineering concepts and constructed rain gardens

OTHER ROLES AND INVOLVEMENTS:

- Private Tutor
- Guiding Eyes for the Blind Puppy Sitter
- Swing Dance Club
- Research Peer Leader
- Summer Undergraduate Research Institute
- Office Assistant
- Columbus Ambassador
- Ohio Academy of Science Member
- Buckeye Readership Competition Winner
- Tau Beta Pi Engineering Futures Program
- Lifeguard and Private Swim Lesson Instructor
- Intramural Sports

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Chapter I - Introduction

Cardiovascular disease and atherosclerosis

Of all health complications, cardiovascular disease is one of the most widespread and pervasive. Atherosclerosis and coronary artery disease, two primary contributors to heart attacks, continue to be the most prominent causes of morbidity and mortality in the United States. They kill over 370,000 people and lead to about 735,000 heart attacks annually according to the Centers for Disease Control and Prevention (CDC)[6]. Cardiovascular diseases are thought to be caused by altercations in the hemodynamic forces applied by blood flows, which induce a variety of shear stresses on the inner surface of blood vessels. Atherosclerotic lesions are often found near bifurcations in arteries, where multidirectional oscillatory flow occurs. Contrarily, straight, healthy arterial portions typically exhibit unidirectional, pulsatile laminar flow. In atheroprone regions, a significant portion of vascular endothelial cells (ECs), the first layer of cells along the inner surface of blood vessels, die via apoptosis, a mechanistic induced cell suicide[12]. Specific intracellular phenomenon responsible for this dysfunction and apoptosis are not fully known. Thus, to further understand, prevent, and eventually treat cardiovascular diseases, the cellular mechanotransduction pathways must be better comprehended.

Role of $[Ca^{2+}]_i$ in NO production via eNOS

To better understand mechanisms that cause dysfunction at the vascular endothelium level, cellular activity during exposures to fluid mechanical shear stress must be explored. Our group, among others, has found that shear stress changes can escalate the oscillations of intracellular calcium concentrations ($[Ca^{2+}]_i$) within the cytosol of cultured ECs (**Figure 1**) [1,2,16,24,30]. Ca^{2+}, a ubiquitous second messenger present in multifarious cellular signaling cascades, is comprehensively studied across scientific disciplines. Through calmodulin, Ca^{2+}

activates endothelial nitric oxide synthase (eNOS), a critical enzyme that produces another important 2nd messenger called nitric oxide (NO)[1,4,10,25]. To maintain vascular health and modulate tone, NO is key since it controls dilation/constriction, which directly impacts blood flow. Increasing shear stress has been proven to increase NO production[10,25]. Yet, indications that NO and reactive oxygen species (ROS) can regulate the $[Ca^{2+}]_i$ response of ECs to different stimuli have been shown[1,3,4,8,9,10].

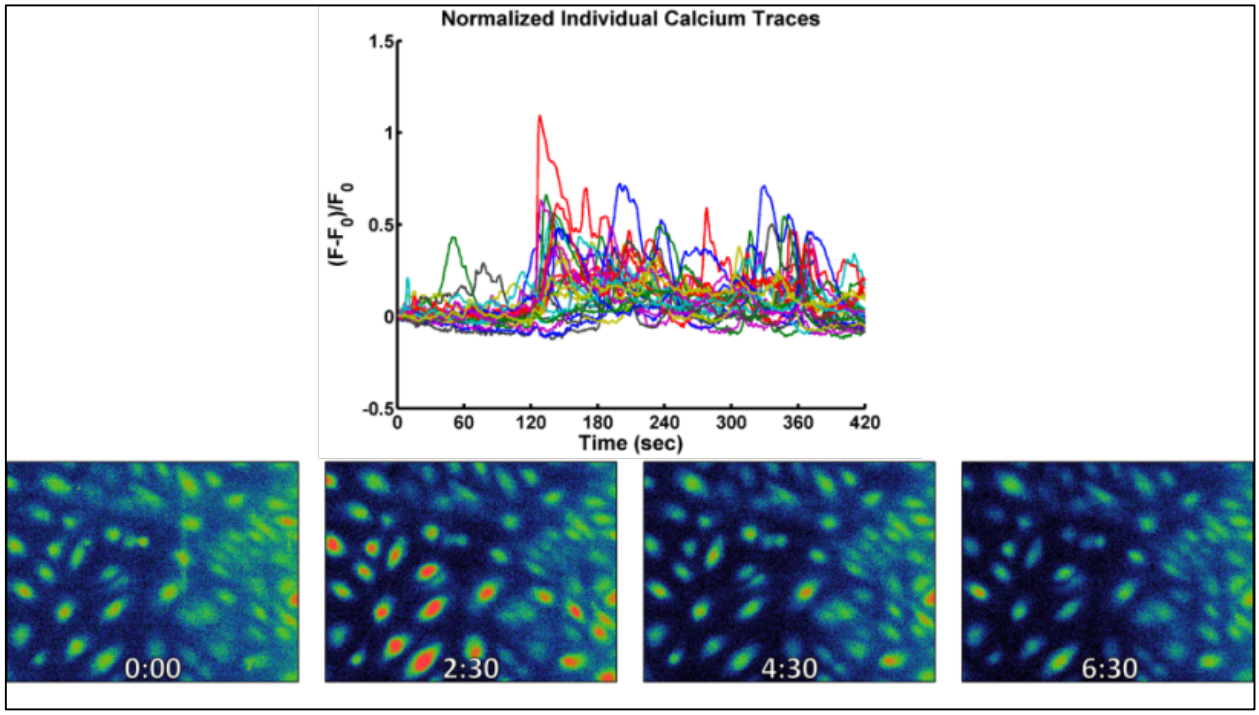

Figure 1. *Endothelial $[Ca^{2+}]_i$ oscillations during laminar shear stress* ***Top***: Fluorescence signals corresponding to Ca^{2+} changes in ECs exposed to flow (10 dynes/cm2). Notice that at 2 min, when flow starts, some ECs respond by transiently increasing their intracellular Ca^{2+} levels. ***Bottom***: Actual fluorescence images (pseudo-colored to show fluorescence intensity – red highest, blue lowest) obtained at different times during flow exposure[23].

Role of shear stress on $[Ca^{2+}]_i$ response

Adenosine triphosphate (ATP) is another critical factor that regulates the EC response to mechanical shear stress[3,15,16,21,22]. Through two pathways, ATP has been shown to elicit responses when ECs were subjected to physiological flow patterns: the P2X and P2Y receptors.

P2X is a direct ion channel that allows extracellular Ca^{2+} influx through the plasma membrane[3,16,21]. The $P2Y_2$ receptor ($P2Y_2R$) channel initiates a cascade with phospholipase C (PLC), generates inositol trisphosphate (IP3), and lastly induces Ca^{2+} efflux via the IP_3R channels of the endoplasmic reticulum (ER), the primary storage center of Ca^{2+} within the cell[3,14,15,20,21]. In our most recent paper, we confirmed that $P2Y_2R$ plays a major, if not the only, role in the shear-induced $[Ca^{2+}]_i$ response[25].

Role of mitochondria on $[Ca^{2+}]_i$ regulation

The mitochondria, the second largest Ca^{2+} store, is often neglected in the $[Ca^{2+}]_i$ response. It is closely localized to the ER IP_3R channels. Due to its adjacent position, a microdomain of $[Ca^{2+}]_i$ considerably higher than the surrounding cytosol is produced when Ca^{2+} is released from the ER. This implies that the mitochondria buffer $[Ca^{2+}]_i$[2,3,15,17,23,25,,26,28,29,30]. Furthermore, other investigators found rises in mitochondrial $[Ca^{2+}]$ when cells were exposed to chemical agonists[11,23,25,28,]. If mitochondria become overloaded with Ca^{2+}, they were found to release all their contents into the cytosol by opening the mitochondrial permeability transition pore (mPTP), including harmful byproducts of the electron transport chain (ETC)[4,11]. In result, a mitochondrial pathway of apoptosis begins, which can potentially induce atherosclerosis initiation/progression.

Importance of EC mitochondria with $[Ca^{2+}]_i$ oscillations

In our most recent paper, we discovered more regarding the effect of laminar flow on $[Ca^{2+}]_i$ mechanisms, especially the importance of the EC mitochondria in enabling $[Ca^{2+}]_i$ transients and oscillations (**Figure 2**). Shear stress, mainly through ATP binding to $P2Y_2R$, is believed to initiate the G protein/PLC/IP_3 pathway. Ca^{2+} is released from the ER when IP_3 activates the IP_3R. At high concentrations, $[Ca^{2+}]_i$ may inhibit or deactivate the IP_3R in the

microdomain between the ER and mitochondria. Additionally, mitochondrial Ca^{2+} uptake through the mitochondrial Ca^{2+} uniporter (MCU) and efflux through the mitochondrial Na^{+}/Ca^{2+} exchanger (mNCX) can regulate the local activation of IP_3R. These interactions heavily influence the first $[Ca^{2+}]_i$ transient and all subsequent oscillations. By releasing Ca^{2+}, mitochondria are known to refill the ER via the sarco-endoplasmic reticulum Ca^{2+}-ATPase channel (SERCA)[25].

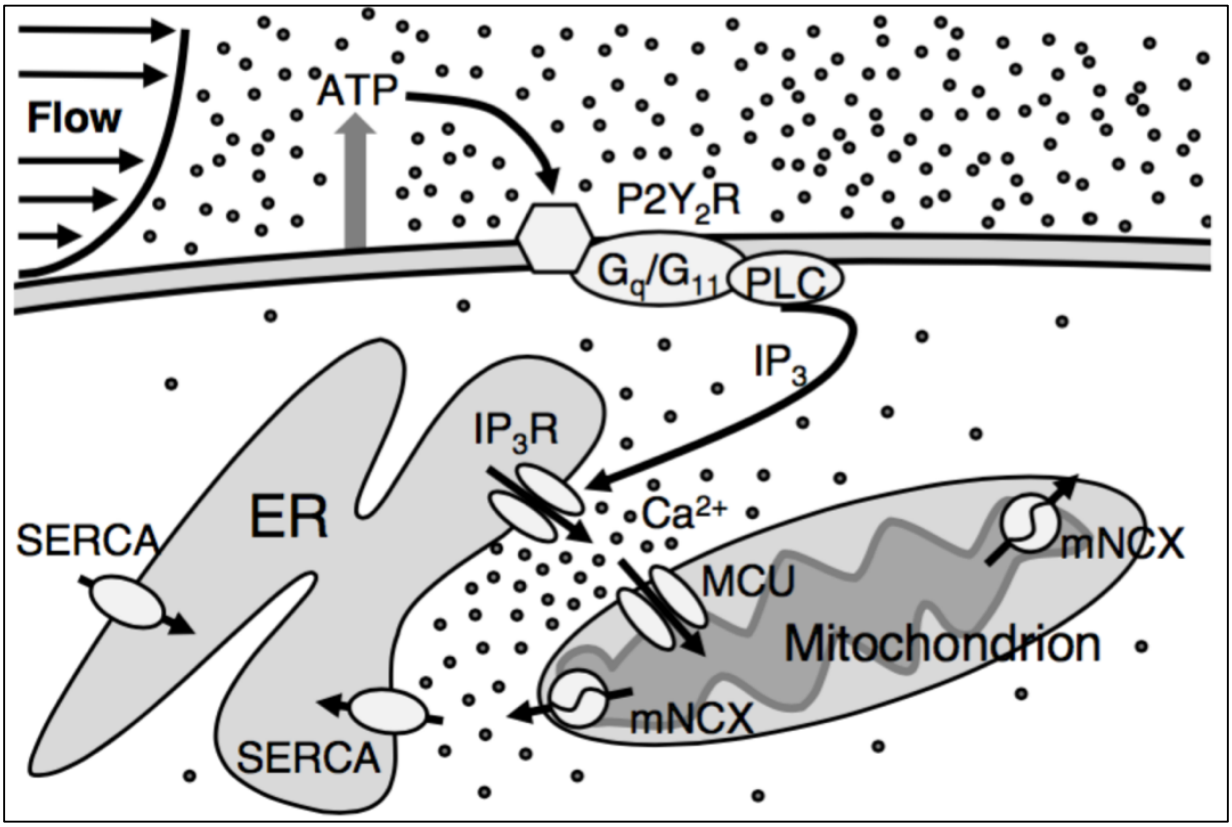

Figure 2. *"Schematic diagram of proposed Ca^{2+} signaling in ECs exposed to shear stress."* Note the EC $[Ca^{2+}]_i$ modulation mechanisms via the Ca^{2+} channels on the ER and mitochondrion initiates by steady laminar shear stress.[24]

Exploring physiological flow profiles

However, our current $[Ca^{2+}]_i$ homeostasis knowledge is based on steady laminar flow profiles (we measured an $[Ca^{2+}]_i$ oscillation frequency of ~20 mHz at a shear stress of 10

dyn/cm^2)[25], which are not physiologically found in arteries. Two physiologically encountered flows are oscillatory and pulsatile flow (**Figure 3**). Oscillatory laminar flow is multidirectional and often present at arterial bifurcations and curvatures where pooling of blood may occur. These regions are characterized by a lack of NO production, increased reactive oxygen species (ROS) production, and increased EC activation, known to contribute to the initiation of atherosclerosis[17]. Pulsatile laminar flow is unidirectional and varying in speed – a product of the pulsing heartbeat. It is typical of straight arterial portions and is atheroprotective in nature due to its proper maintenance of NO and ROS production[12]. To embark toward more physiological flows, this study aims to expand upon our earlier published work by investigating the shear-induced $[Ca^{2+}]_i$ response of cultured ECs to pulsatile and oscillatory laminar flows; we hypothesize that pulsatile and oscillatory laminar flows will produce different shear-induced $[Ca^{2+}]_i$ responses. Better understanding of EC Ca^{2+} signaling may lead to discovery of better prevention and/or treatment strategies for patients with cardiovascular disease.

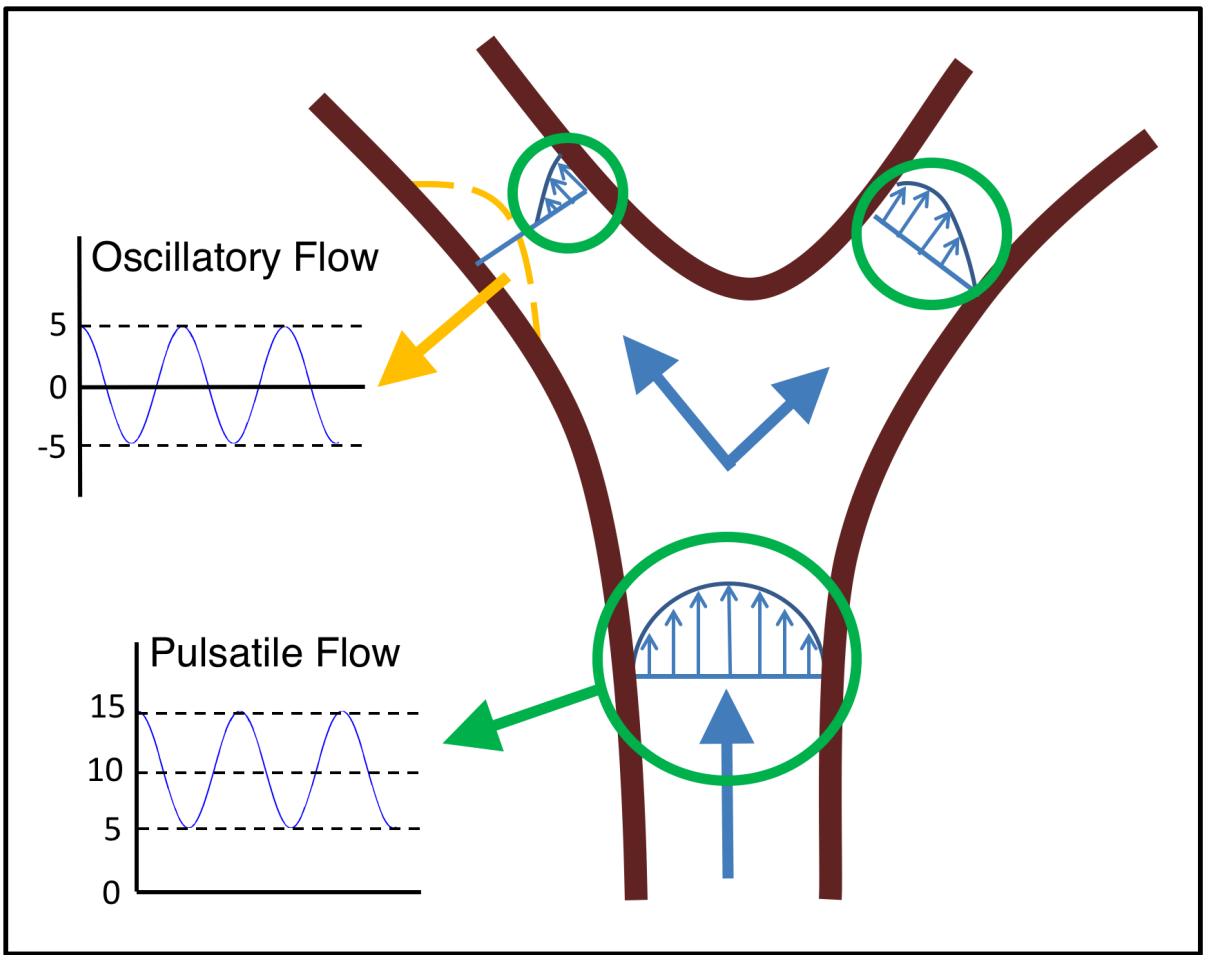

Figure 3. *Schematic diagram of an arterial bifurcation.* It portrays: 1) a physiological, unidirectional pulsatile flow (10±5 dyn/cm^2) denoted in green and 2) a pathological, multi-directional oscillatory flow (0.1±5 dyn/cm^2) in yellow. Each flow induces mechanical shear stress on ECs that elicits intracellular Ca^{2+} responses.

Chapter II - Materials and Methods

EC Preparation

Parallel-plate perfusion chambers (Ibidi μ-slide $IV^{0.4}$) were seeded with cells from a human umbilical vein EC (HUVEC) line, EA.hy926. Upon confluency, the ECs were preincubated in a saline buffer (HBSS with 20 mM HEPES) for 40 min. Next, ECs were incubated with a Ca^{2+} fluorophore (3μM Fluo-4 AM) for 20 min to fluorescently label their $[Ca^{2+}]_i$. Lastly, ECs were rinsed with saline buffer before applying any shear stresses.[25]

Shear Stress Application

A gas tight syringe was filled with saline buffer and connected to an automated syringe pump (Harvard Apparatus) programmed to generate specific flow rates. An initial 2 min period of static incubation preceded a 5 min period of fluid shear stress with saline buffer. Four types of shear stress treatments were applied:

- Pulsatile flow: 10±5 dyn/cm^2, 1 Hz
- Oscillatory flow: 0.1±5 dyn/cm^2, 1 Hz
- Static, no flow (control): 0 dyn/cm^2, constant

The 1 Hz designates how often the syringe pump cycled through the entire flow profile. A zero flow profile was maintained during the static (no flow) trials. Due to syringe pump limitations, flows were not applied in a smooth cosine-like waveform as in physiological flows, but were approximated (**Figure 4**). The 1 Hz interval would involve 0.5 sec of the highest flow followed by 0.5 sec of the lowest flow to average to the mean value. This cycle was repeated each sec for the entire 5 min shear period. For example, in pulsatile flow 0.5 sec of 15 dyn/cm^2 shear stress was followed by 0.5 sec of 5 dyn/cm^2 to create a full flow cycle.

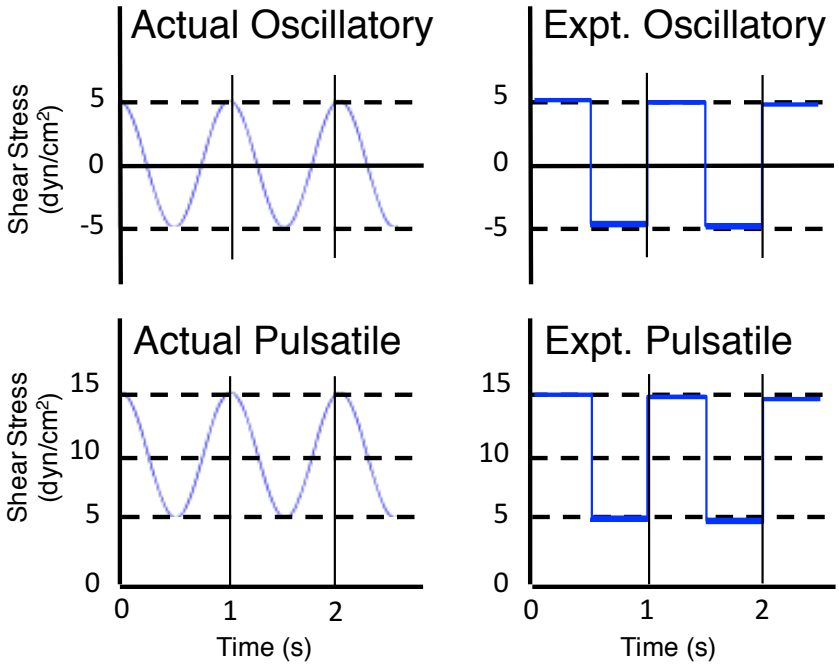

Figure 4. *Physiological shear stress approximation*. The plots on the left (actual oscillatory and pulsatile flow) both crudely describe the shear stress profile of physiological flows. Plots on the right (experimental oscillatory and pulsatile flow) both represent the actual shear stress profiles applied to a monolayer of HUVECs used to approximate actual flows.

Fluorescence Microscopy and Digital Processing

Fluorescence microscopy (Nikon; 494 nm excitation/506 nm emission; 20x magnification) was utilized to image fluorescence changes each second to generate a final 7 min video of the $[Ca^{2+}]_i$ responses. Videos were saved as uncompressed .avi files to avoid any loss in resolution. Normalized fluorescence per pixel in arbitrary units was calculated and compared from each individual image for all videos. Percentages of responding and oscillating cells, peak magnitude, and oscillation frequency of each cell in the field of view were quantified using ImageJ, CellProfiler and an in-house MATLAB code, all of which are described in **Figure 5**. A peak was counted any time a change in sign of the normalized fluorescence time derivative occurred with an increase ≥15% in normalized fluorescence.

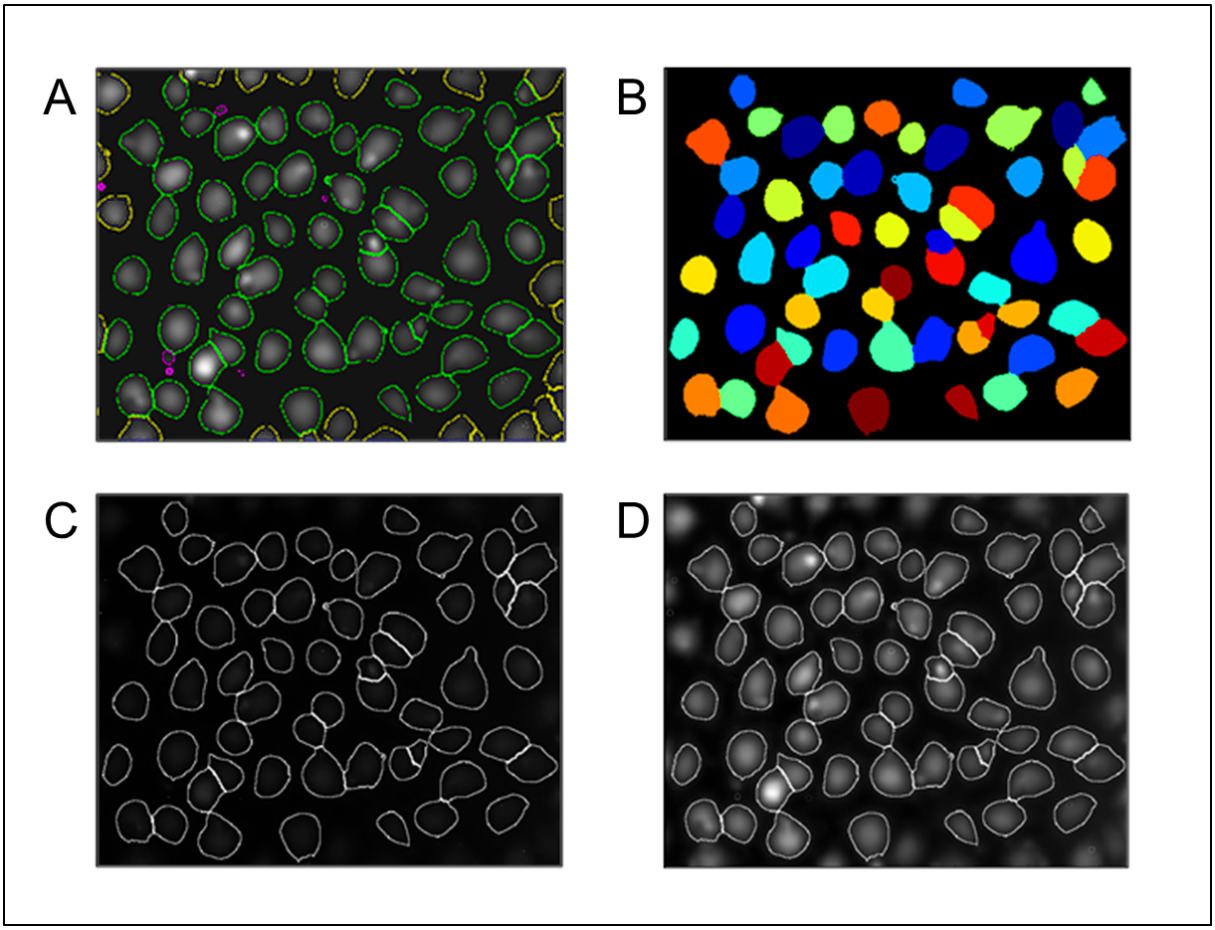

Figure 5. *Digital processing of fluorescence images.* The following process was employed to prepare the images for MATLAB analysis. *A)* The original image was obtained by performing a z-stack time average of video frames. It was despeckled to improve efficacy of thresholding and partitioned using watershed segmentation. *B)* Regions were outlined based on an intensity derivative. Regions touching the periphery of the image or those that are too small were removed. *C)* Region outlines were overlaid on enhanced images. *D)* Region outlines were placed over the original images to verify that the program correctly outlined each cell.

Statistical Analysis

Measured parameters of responding cells (%), peak magnitude, oscillating cells (%), and oscillation frequency (mHz) were shown as means ± standard errors. One-way ANOVA tests and Tukey's post hoc analysis were used to compare the data among different shear profiles

(static, pulsatile, oscillatory). In the plot of responding cells (%) vs. time (min), one-way ANOVA tests and paired *t*-tests with a Bonferroni-Holm correction were run at each time point. JMP statistical software (SAS Institute) was used for all analysis. Confidence intervals of 95% or greater were considered significant.[25]

Chapter III – Results and Discussion

Pulsatile Flow vs. Static Control

During pulsatile flow, ECs produced a synchronized fluorescence response at the onset of shear (**Figure 6A**), and almost all of the cells (> 90%) responded, or peaked in normalized fluorescence, during the 1st min after shear onset (**Figures 7 and 8A**). Approximately 10% of ECs produced more than one transient during the 5 min treatment (**Figure 8B**). These ECs were deemed "oscillating cells" since they responded more than once. In comparison to the static condition, the % responding cells, peak magnitude, and oscillation frequency all differed significantly. The static control yielded no $[Ca^{2+}]_i$ transients.

Oscillatory Flow vs. Static Control

During oscillatory flow, ECs produced a fluorescence response after the onset of shear (**Figure 6B**), and the majority of cells (60%) responded at some point during the 5 min shear stress portion (**Figures 7 and 8A**). Notably, almost 40% of ECs oscillated during the 5 min treatment (**Figure 8B**). In comparison to the static condition, the % responding cells, % oscillating cells, peak magnitude, and oscillation frequency all differed significantly. Again, the static control yielded no $[Ca^{2+}]_i$ transients.

Oscillatory vs. Pulsatile Flow

Both flows produced $[Ca^{2+}]_i$ responses during the 5 min application of shear stress, but there were notable differences in their nature.

During the initial response at the onset of shear, significant discrepancies occurred. Only 20% of ECs under oscillatory flow responded while over 90% did during pulsatile flow (**Figure 7**). The large peak in the average normalized fluorescence supports this synchronization since

the majority of cells expressed strong transients. Pulsatile flow average peak magnitude was considerably greater than the practically negligible oscillatory average peak magnitude, too (**Figures 6A, 8**). Differences in shear stress average values (10±5 vs. 0.1±5 dyn/cm^2) could be attributed to these findings. Though, the lack of a synchronized first peak in oscillatory flow could potentially lead to more spontaneous and disorganized responses throughout the shearing process, which is evident during oscillatory flow and minimal during pulsatile. Thus, shear stress patterns seem to dictate the magnitude and temporal synchronization of $[Ca^{2+}]_i$ transients.

Throughout the entire 5 min shear period, important disparities existed regarding % responding and % oscillating cells. In **Figure 7**, the % responding cells under pulsatile flow dramatically reduced (90% to < 10%) each minute after onset while oscillatory flow maintained a consistent response rate per minute (15-35%). The % oscillating cells was significantly higher under oscillatory flow (~40%) than pulsatile (~10%) (**Figure 8B**). A probable cause of these differences was the alternating directionality of the oscillatory flow, and most importantly the net forward flow under pulsatile flow. Specifically, ECs under pulsatile flow experienced unidirectional shearing. Meanwhile, ECs under oscillatory flow had fluid shear forward and backward with little net forward movement, which resulted in the heightened quantity of $[Ca^{2+}]_i$ oscillations. Thus, the direction of shear stress is likely the initiator. Cellular mechanisms responsible are still under investigation, but the PLC/IP_3R pathway is a probable source of Ca^{2+} for transients and oscillations.

Additionally, under oscillatory flow, the cells that responded were more likely to experience $[Ca^{2+}]_i$ oscillations. Over 60% of the responding cells oscillated; meanwhile only ~10% of responding cells oscillated under pulsatile flow (**Figure 8A, 8B**). This increased $[Ca^{2+}]_i$ release is significant. This could be due to the fact that peak magnitudes were lower in

oscillatory vs. pulsatile flow (**Figure 8C**). Smaller peak magnitudes indicate that less Ca^{2+} was released from the ER initially, meaning more Ca^{2+} is retained in the ER for another efflux. The ER of ECs under pulsatile flow would require more time to reuptake and replenish its Ca^{2+}. Other factors could be responsible for this difference as well, but overall the application of oscillatory flow seems to increase the EC $[Ca^{2+}]_i$ activity.

Pulsatile and Oscillatory Flow vs. Static Control

In both flow treatments, little to no responses were observed during the 2 min static period (**Figure 8**), which is expected since no shear stress is present to trigger the IP_3R pathway. This supports the assumption that no exogenous factors were influencing the $[Ca^{2+}]_i$ responses aside from the application of shear stress during experimentation.

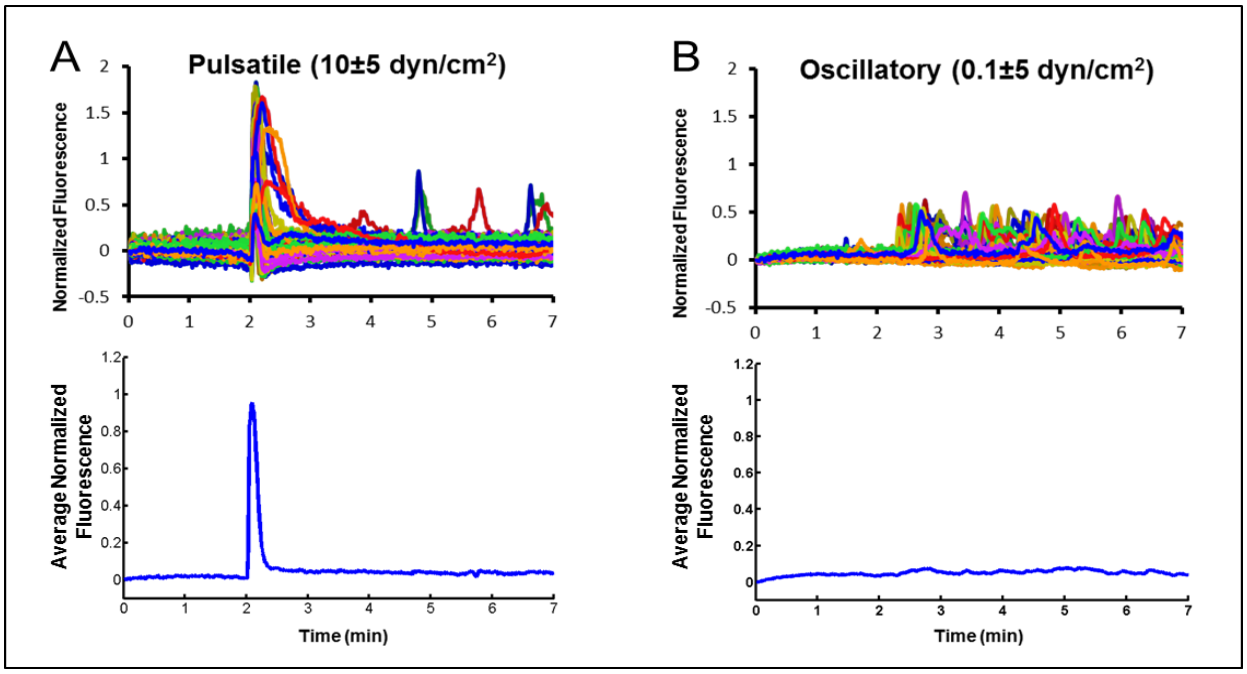

Figure 6. *Characteristic normalized fluorescence changes during EC exposure to shear stress*. In the top graphs, each colored line represents an individual cell's fluorescence over time. In the bottom graphs, the normalized fluorescence data from all cells at each time point in the field of view are averaged into one plot. *A)* Pulsatile flow demonstrates a single synchronized response to the onset of flow with few subsequent oscillations. *B)* Oscillatory flow portrays responses of lower magnitude without any synchronization and oscillations throughout.

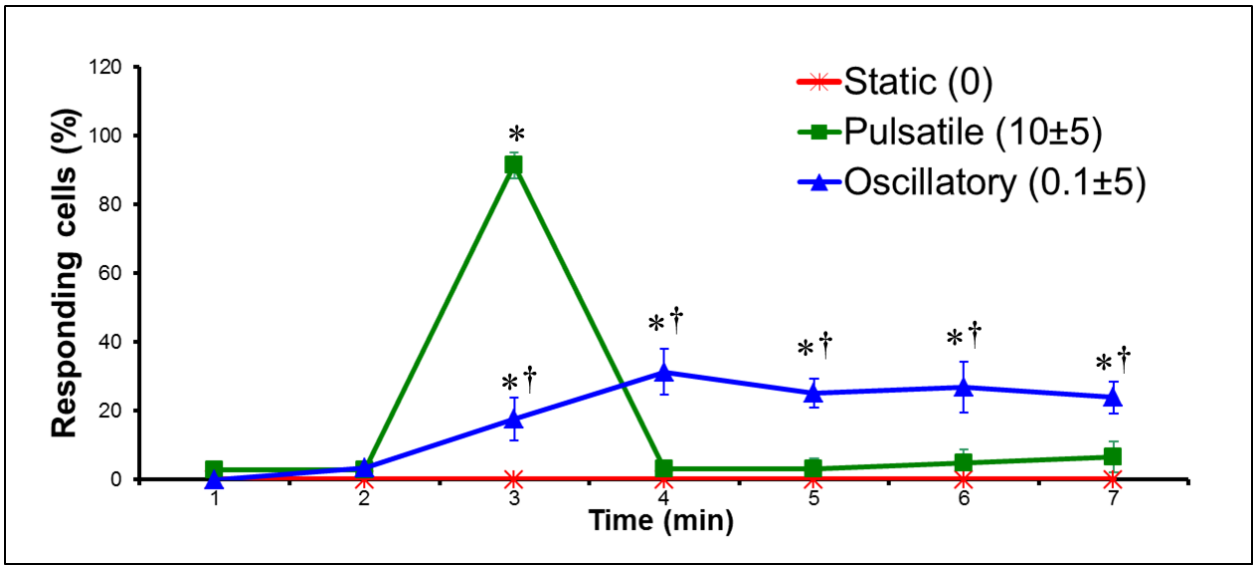

Figure 7. *Shear-induced $[Ca^{2+}]_i$ transients in ECs under different flows.* Responding cells (%) at the end of each minute) during either a 7-min static period or a 2-min static period followed by a 5-min shear period. Data are means±SE (n=3-4 experiments). *P < 0.05 vs. pre-shear static period. †P < 0.05 vs. pulsatile.

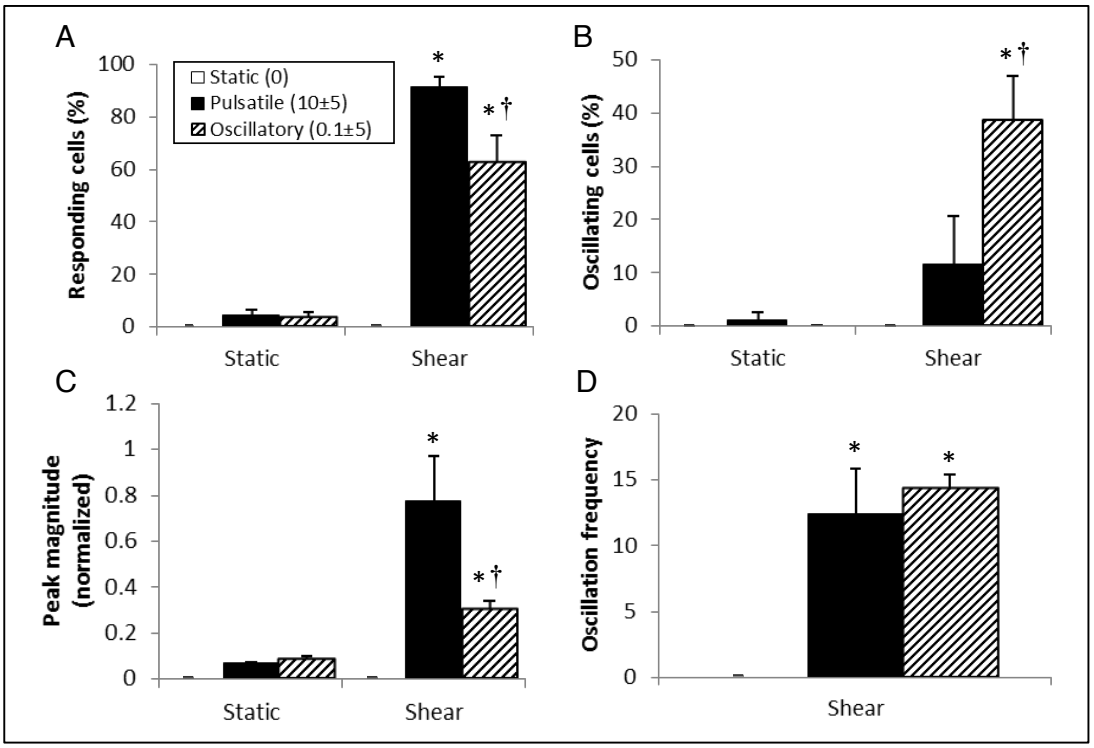

Figure 8. *Shear stress dependently changes key measures of the $[Ca^{2+}]_i$ response. A)* Responding cells (%) at the end of 2-min static vs. 5-min flow. *B)* Oscillating cells (%) at the end of static vs. flow. *C)* Largest peak averaged over responding cells per experiment and across experiments. *D)* Oscillation frequency of oscillating cells at different flows. Data are means±SE (n = 3–4 experiments). *P < 0.05 vs. static. †P < 0.05 vs. pulsatile.

Significance of differing $[Ca^{2+}]_i$ signals

Agonists are known to produce $[Ca^{2+}]_i$ transients and oscillations in cells of all types. Oscillations in non-excitable cells, such as ECs, are mainly controlled by IP_3 and $[Ca^{2+}]_i$ which modulate the IP_3R activity on the ER. $[Ca^{2+}]_i$ has a biphasic effect on IP_3R activity[18,27]. In small concentrations, it initiates a Ca^{2+} transient; in large concentrations, it is inhibitory of a Ca^{2+} transient. In comparison to sustained elevations or transients, some believe that $[Ca^{2+}]_i$ oscillations are beneficial to prevent Ca^{2+} overload and desensitization. As for the transfer of information via $[Ca^{2+}]_i$, oscillations, they provide a much more diverse and variable range of responses versus a single sustained transient. Changes in the amplitude, frequency, duration, and other parameters can all impart unique responses within the cell[18,28]. Frequency, in particular, variably activates certain decoder proteins such as the nuclear factor (NF)-κB or Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)[15,27]. CaMKII activates eNOS to produce NO, which ultimately regulates EC functionality.

Between pulsatile and oscillatory flows administered during this experiment, differences in amplitude, oscillating cell (%), and responding cells (%) were found. However, no significant difference was seen between the oscillation frequency. Oscillatory flow demonstrated a significantly higher number of oscillating cells (~40%) than pulsatile (~10%).. Future studies of actual NO/ROS production are needed to better interpret our findings and connect them with cell functional assays. Based on the literature, oscillatory flow produces more ROS and less/no NO than pulsatile flow, which would suggest that the higher % of oscillating ECs under oscillatory flow may be experiencing increased ROS levels.

Overall, knowing a difference in $[Ca^{2+}]_i$ exists indicates that there is likely a difference in the types of EC signaling responses. Consequentially, this can impart varying activation of

mechanosensitive kinases and transcription factors that affect NO production. Without adequate concentrations of NO, an imbalance of ROS can accumulate and shift the cell toward an inflammatory or apoptotic state (this is thought to be the case under oscillatory flow)

As for the $[Ca^{2+}]_i$ synchronization response expressed during the initial onset of pulsatile shear stress, it may hold high significance in a study of arterial reperfusion after ischemia (where the flow changes abruptly from zero to arterial levels). Since ECs under physiological conditions do not experience a new onset of flow (always experiencing a flow), data during the first minute may not be representative of the cell response to the applied flow profile. More in depth analysis of the post-onset periods (3-7 min) should be conducted to compare these flows.

Chapter IV – Conclusion

Motivation of Study and Conclusions

By understanding how ECs respond to fluid shear stresses experienced by changing flow patterns in the vasculature, we will increase our knowledge on factors that affect the health of human blood vessels. Understanding $[Ca^{2+}]_i$ can lead to a better knowledge of its effect on NO/ROS balance, which can potentially unveil causes of cellular dysfunction/apoptosis and initiation/progression of cardiovascular disease. Through this work, evident differences in EC $[Ca^{2+}]_i$ responses were found between oscillatory flow (typical of atheroprone areas in human arteries), and pulsatile flow (typical of atheroprotective areas in human arteries). In oscillatory vs. pulsatile flow, lower $[Ca^{2+}]_i$ peak magnitudes without initial synchronization, higher % of oscillating cells, and lower % of responding cells was observed. Additionally, the characterization of the EC $[Ca^{2+}]_i$ activity under these flow types may have laid the foundation for future investigations.

Future Directions

With upcoming work, we need to better understand the role of the ER, mitochondria, and extracellular Ca^{2+} on EC $[Ca^{2+}]_i$ activity under different flows. We currently only know this relationship under steady laminar flow[26]. Investigating the signaling that regulates the opening of the IP_3R and/or MCU under oscillatory and pulsatile flow could reveal more on such a relationship. Additionally, our current pump creates abrupt increases and decreases (square waves) in flow/shear stress, which do not correspond to real arterial flow profiles during the cardiac diastole and systole. While the pulsatile and oscillatory flows tested are more realistic than steady laminar flow, they are still not fully representative of the profiles produced by the cardiac cycle.

Additionally, based upon the data presented, the initial EC $[Ca^{2+}]_i$ response to the onset of shear is not typical of physiological flows unless in a rare situation, such as reperfusion following ischemia. Thus, further analysis and comparison of the $[Ca^{2+}]_i$ responses beyond the onset of reperfusion period should be examined to better approach a true physiological model of consistent blood flow in an artery.

Currently, our group is working to calibrate the fluorescence signals via a Ca^{2+} standard curve. This will enable the fluorescence signals to be converted to actual micromolar Ca^{2+} concentrations for better understanding and direct comparisons among different runs and flow profiles. These values will also be advantageous for evaluating and improving mathematical models of the EC $[Ca^{2+}]_i$ mechanisms that our laboratory and collaborators are currently developing.

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